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Optimizing the Aggregation Propensity of Therapeutic Monoclonal Antibodies against Cancer and Autoimmune Diseases: A Computational Study

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AIGR, VPG and NCP collected the data, performed calculations and evaluated the results. Author SJH supervised the whole project and evaluated the results. All authors contributed to the writing of the manuscript. All authors have read and approve the final version of the manuscript.

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ABSTRACT

Monoclonal antibodies (mAbs) represent the most promising and rapidly growing class of therapeutic compounds for treating a wide variety of human chronic and acute diseases. Despite their benefits, a major drawback in the exploitation of antibodies is their tendency to form aggregates. As a result, severe immunological reactions in patients have been recorded.
In this study, we investigated the susceptibility of a set of therapeutic monoclonal antibodies to form aggregates. We selected antibodies that have all been approved by the U. S. Food and Drug Administration and are indicated for the treatment of various types of cancer and autoimmune diseases. The AMYLPRED 2 consensus method was used to predict 'aggregation-prone' regions on the surface of these proteins.

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These regions are conserved and observed in almost all monoclonal antibodies commercially available. Considering the amino acid sequences of these antibodies, common groups of 'aggregation-prone' regions were identified, called clusters. We successfully reduced or even fully eliminated 'aggregation-prone' groups (clusters) by specific 'mutations' of the amino acids with exposed side chains. This information may be useful in future studies of monoclonal antibodies by improving existing therapeutic products or by designing novel ones.

Keywords: Aggregation-prone regions; monoclonal antibodies; aggregation propensity; cancer; autoimmune diseases.

ABBREVIATIONS

mAbs, Monoclonal Antibodies; CDRs, Complementarity Determining Regions; FDA, U.S. Food and Drug Administration; L1, Light chain CDR1; L2, Light chain CDR2; L3, Light chain CDR3; H1, Heavy chain CDR1; H2, Heavy chain CDR2; H3, Heavy chain CDR3.

1. INTRODUCTION

Monoclonal antibodies (mAbs) are tetramers of two identical pairs of polypeptide chains, the heavy and the light chains [1]. The light chain consists of one variable and one constant domain and the heavy chain consists of one variable and three or more constant domains [1]. All the domains have a similar tertiary structure called immunoglobulin fold, which is characterized by two anti-parallel beta sheets [2]. The antigen-binding site consists of six loops, three from the light chain and three from the heavy chain [3]. These loops are also known as complementarity determining regions (CDRs) and they are responsible for the recognition and the binding between antibody and antigen [4].

Given almost any substance, it is possible to produce monoclonal antibodies that specifically bind to it [5]. MAbs are made by identical immune cells that are clones of a unique parent B-cell, have monovalent affinity, bind to the same epitope and can be reproduced endlessly *in vitro* [6].

They are used in treatment schemes for autoimmune [7], cardiovascular [8] and infectious diseases [9], cancers [10,11] and others. They can also be used as diagnostic [12] and biotechnological tools [13].

Monoclonal antibodies in high concentrations, in their storage and usage, form aggregates [14], which are an increasing concern in therapeutic development. Protein aggregation causes side effects and immunogenic responses in clinical trials [15]. Aggregates may also result in reduction of antibody therapeutic activity, while severe immunological reactions in patients have been recorded [16]. Thus, it is important to understand the aggregation mechanisms of mAbs under different circumstances [17] and their potential biological implications in the pharmaceutical industry in order to improve their efficacy and make them successful biopharmaceutical products [18].

Several computational studies have been carried out and a variety of monoclonal antibodies have been extensively studied and engineered [19]. A number of computational tools are also available [20] to predict aggregation-prone regions in therapeutic proteins. These tools focus on predicting the aggregation-prone regions (small peptides) and finding the potential sequence and structure factors that contribute toward aggregation formation in proteins [21]. As a result, a number of monoclonal antibodies have been improved, used again and for other cases too.

Taking into account all these insights, our purpose was to propose novel models of therapeutic monoclonal antibodies with optimized aggregation propensity, based on an amino acid substitution approach. Our findings can be applied on rational design of novel therapeutic candidates or improvement of existing bio-therapeutics.

2. MATERIALS AND METHODS

2.1 Dataset Collection

In order to study the monoclonal antibodies, we carried out an extensive research in the literature and available databases. We analyzed antibodies that have been approved by the U.S. Food and Drug Administration (FDA) and have

been indicated for the treatment of various acute and chronic diseases like cancer and autoimmune diseases. They are all IgG1 kappa immunoglobulins and they have several and different mechanisms of action.

We collected the amino acid sequences of antibodies from the Protein Data Bank [22]. We obtained also the three-dimensional structures of antibodies from the Protein Data Bank (PDB ID for Alemtuzumab: **1CE1**, PDB ID for Bevacizumab: **1BJ1**, PDB ID for Cetuximab: **1YY8**, PDB ID for Ofatumumab: **3GIZ**, PDB ID for Pertuzumab: **1L71**, PDB ID for Rituximab: **2OSL**, PDB ID for Trastuzumab: **1N8Z**, PDB ID for Adalimumab: **3WD5**, PDB ID for Infliximab: **4G3Y**, PDB ID for Natalizumab: **4IRZ**, PDB ID for Ustekinumab: **3HMX**).

Additional information about the monoclonal antibodies was obtained from the Drug Bank [23] and the Monoclonal Antibodies Database, part of the IMGT®, the international ImMunoGeneTics information system® [24] in order to perform sequence analysis.

2.2 Monoclonal Antibodies against Cancer

Detailed information about monoclonal antibodies against cancer used in this study is presented in Table 1.

2.3 Monoclonal Antibodies against Autoimmune Diseases

Detailed information about monoclonal antibodies against autoimmune diseases used in this study is presented in Table 2.

2.4 Aggregation-prone Regions

In order to predict 'aggregation-prone' regions on the surface of antibodies, we used AMYLPRED2 [25], a consensus method, which predicts 'aggregation-prone' peptides on an amino acid sequence, from sequence alone, developed in our lab. For each monoclonal antibody, the algorithm was applied to the amino acid sequence of each chain separately.

Furthermore, sequence alignments of heavy chains and light chains were performed utilizing ClustalW [26], to identify similarities and differences between the antibodies used.

2.5 Criteria of the Amino-acid Mutation

Three criteria were followed in order to select the amino acids that may be substituted by others in the amino acid sequences of the monoclonal antibodies of the dataset.

The first criterion was the accessibility of these amino acids to the solvent in the 3D-structure. It is undesirable to change amino acids that are not on the surface of the protein, because these changes will probably affect the 3D-structure and the stability of the molecule. For this reason, we located the amino acid residues of the antibodies exposed to the solvent with the help of the DSSP algorithm [27]. Residues that are in contact with at least two molecules of water and they are predicted by AMYLPRED2 [25] to be in 'aggregation-prone' regions, were selected as possible candidates to be substituted by others.

The second criterion was that the residues to be substituted should not be found in CDRs. Residues in these regions are responsible for the recognition and the interaction with the antigen-target. For these reason, any change in these regions may cause unexpected effects to the functionality of the antibody.

The third criterion was to replace amino acid residues with exposed side chains, in the predicted 'aggregation-prone' regions, considering the experimental aggregation propensities of the 20 natural amino acids [28]. According to the propensities, residues that are 'aggregation-prone' have positive values and those that are not have negative values. The higher the tension to form aggregates, the greater the positive value is and the same applies for the negative values, respectively.

We also took into account the 3D-structure (Fab fragment) of the antibodies in order to be certain that our 'mutations' do not affect overall the 3D-structure.

2.6 Clusters

After performing sequence alignments of all the monoclonal antibodies in the dataset, for the light (Fig. 1) and the heavy (Fig. 2) chain separately, we discovered some common groups of 'aggregation-prone' regions in the amino acid sequences of the antibodies that are present in almost all the monoclonal antibodies that are now on the market, against cancer and autoimmune diseases, in a similar way as it was done in [29].

Table 1. Monoclonal antibodies against cancer used in this study

	MAbs	Trade name	Source	Type	Target	PDB Id	Clinical indications	FDA approval	Clinical trials
1	Alemtuzumab [42-47]	CAMPATH®	Humanized	IgG1-kappa	CD52	1CE1, 1BEY	Chronic lymphocytic leukemia	2001	Phase M
							Multiple sclerosis	2013	Phase M
							Kidney transplant rejection		Phase I/II
2	Bevacizumab [48-53]	AVASTIN®	Humanized	IgG1-kappa	VEGF-A	1BJ1	Colorectal cancer	2004	Phase M
							Lung cancer	2006	Phase M
							Breast cancer	2008	Phase M
							Renal cell carcinoma	2009	Phase M
							Glioblastoma	2011	Phase M
							Cervical carcinoma	2014	Phase M
3	Cetuximab [54-59]	ERBITUX®	Chimeric	IgG1-kappa	EGFR	1YY8, 1YY9	Colorectal cancer	2004	Phase M
4	Ofatumumab [60-65]	ARZERRA®	Human	IgG1-kappa	CD20	3GIZ	Chronic lymphocytic leukemia	2009	Phase M
							Non-Hodgkin’s lymphoma		Phase III
5	Pertuzumab [66-68]	PERJETA®	Humanized	IgG1-kappa	ERBB2	1L7I, 1S78	Breast cancer	2012	Phase M
							Prostate cancer		Phase II
6	Rituximab [69-74]	RITUXAN®	Chimeric	IgG1-kappa	CD20	2OSL	Non-Hodgkin’s lymphoma	1997	Phase M
							Chronic lymphocytic leukemia	2010	Phase M
7	Trastuzumab [75-79]	HERCEPTIN®	Humanized	IgG1-kappa	ERBB2	1N8Z	Breast cancer	1998	Phase M
							Gastric cancer	2010	Phase M

Table 2. Monoclonal antibodies against autoimmune diseases used in this study

	MAbs	Trade name	Source	Type	Target	PDB Id	Clinical Indications	FDA approval	Clinical trials
1	Adalimumab [80-87]	HUMIRA®	Human	IgG1-kappa	TNF-A	3WD5	Rheumatoid arthritis	2002	Phase M
							Psoriatic arthritis	2005	Phase M
							Ankylosing spondylitis	2006	Phase M
							Juvenile idiopathic arthritis	2008	Phase M
							Psoriasis	2008	Phase M
							Crohn’s disease	2010	Phase M
							Ulcerative colitis	2012	Phase M
2	Infliximab [88-95]	REMICADE®	Chimeric	IgG1-kappa	TNF-A	4G3Y	Crohn’s disease	1998	Phase M
							Rheumatoid arthritis	1999	Phase M
							Ankylosing spondylitis	2004	Phase M
							Ulcerative colitis	2005	Phase M
							Psoriatic arthritis	2005	Phase M
							Psoriasis	2006	Phase M
3	Natalizumab [96-98]	TYSABRI®	Humanized	IgG4	ITGA4	4IRZ	Multiple sclerosis	2004/2006	Phase M
							Crohn’s disease	2008	Phase M
4	Rituximab [69-74]	RITUXAN® ABTHERA®	Chimeric	IgG1-kappa	CD20	2OSL	Rheumatoid arthritis	2006	Phase M
5	Ustekinumab [99-103]	STELARA®	Human	IgG1-kappa	IL12A L12B	3HMX 3HMW	Psoriasis	2009	Phase M
							Psoriatic arthritis	2013	Phase M
							Multiple sclerosis		Phase II
							Crohn’s disease		Phase II

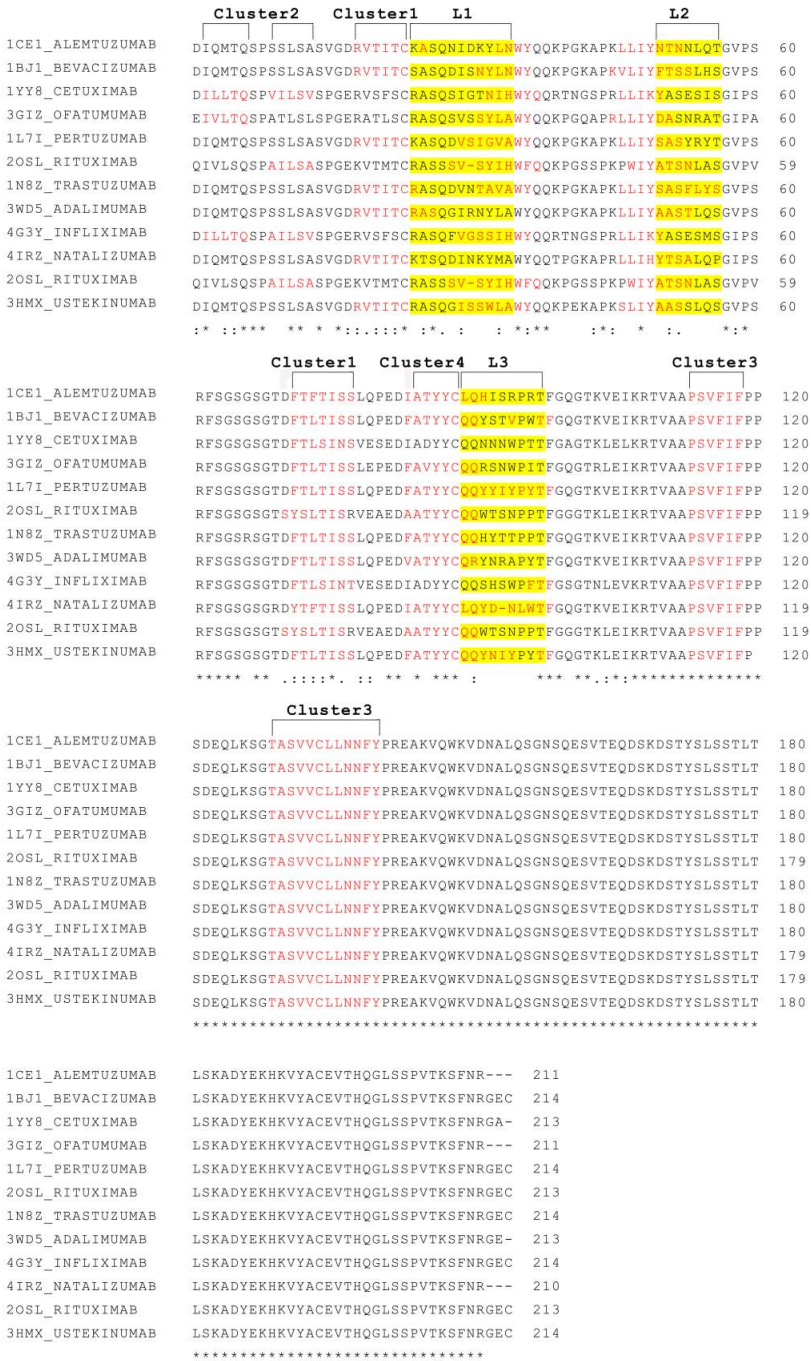

Fig. 1. Sequence alignment of light chains of all the monoclonal antibodies of the dataset

ClustalW was used for the alignment [26]. The molecule name and the PDB ID are mentioned. The three CDRs are highlighted in yellow and named L1, L2 and L3. The predicted aggregation-prone regions are shown in red. The clusters of 'aggregation-prone' regions are also indicated (Cluster1, Cluster2, Cluster3, Cluster4)

These 'aggregation-prone' regions were grouped into clusters according to their positions in the 3D-structure (Figs. 3A, B). With specific changes of amino acid residues, only in the peptides that are present in 'aggregation-prone' regions, we successfully diminished 'aggregation-prone' clusters or even made them to disappear completely (Figs. 3A, B).

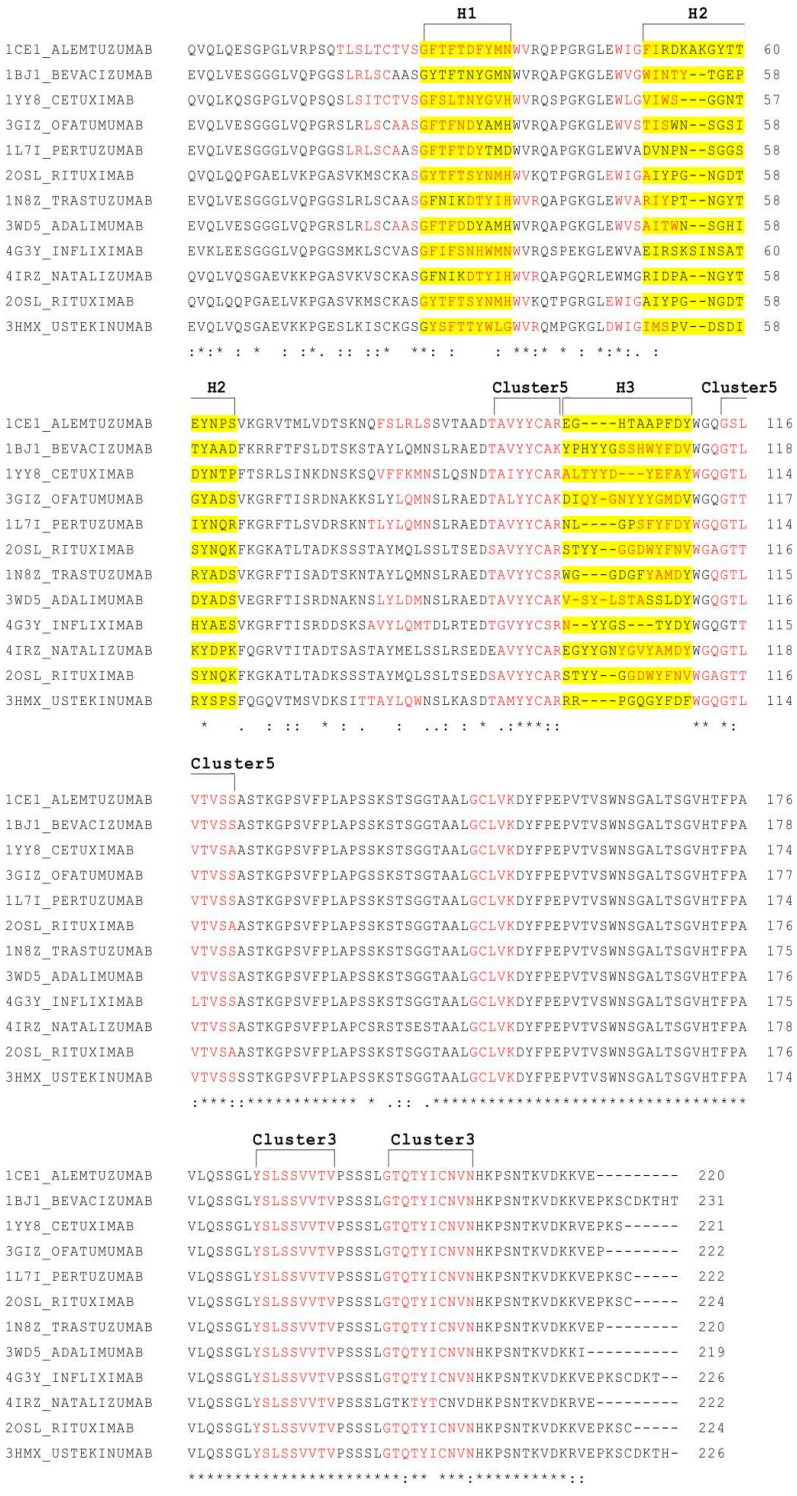

Fig. 2. Sequence alignment of heavy chains of all the monoclonal antibodies of the dataset

ClustalW was used for the alignment [26]. The molecule name and the PDB ID are mentioned. The three CDRs are highlighted in yellow and named H1, H2 and H3. The predicted aggregation-prone regions are in red. The clusters of 'aggregation-prone' regions are also indicated (Cluster3, Cluster5)

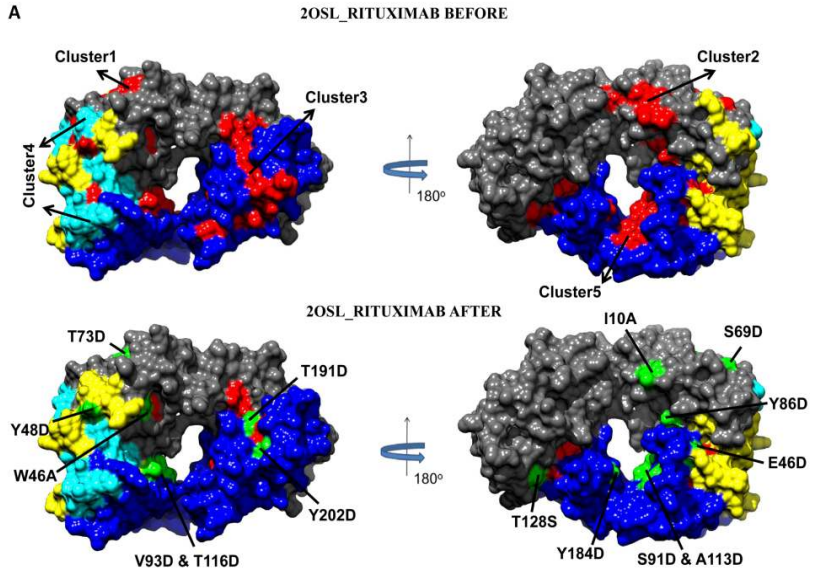

Fig. 3A. Space-filling models of the monoclonal antibody Rituximab (Rituxan) (PDB ID: 2OSL) produced utilizing the software Chimera 1.8 [31]

The molecule name and the PDB ID are mentioned. Light chain in grey, heavy chain in blue, 'aggregation-prone' regions in red, CDRs in yellow, 'aggregation-prone' regions in CDRs in cyan and the residues that will be substituted are depicted with green color. The 'BEFORE' images illustrate the stage before the substitutions and the names of the clusters, for front and back view. The 'AFTER' images represent the structures after the substitutions and the substitutions made (residue that was substituted and position), for front and back view

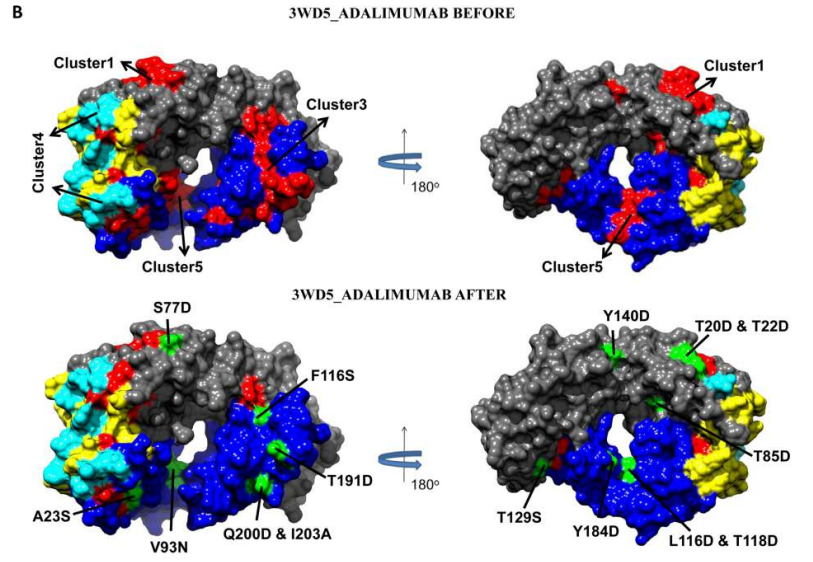

Fig. 3B. Space-filling models of the monoclonal antibody Adalimumab (Humira) (PDB ID: 3WD5) produced utilizing the software Chimera 1.8 [31]

The molecule name and the PDB ID are mentioned. Light chain in grey, heavy chain in blue, 'aggregation-prone' regions in red, CDRs in yellow, 'aggregation-prone' regions in CDRs in cyan and the residues that will be substituted are depicted with green color. The 'BEFORE' images illustrate the stage before the substitutions and the names of the clusters, for front and back view. The 'AFTER' images represent the structures after the substitutions and the substitutions made (residue that was substituted and position), for front and back view

The procedure of selecting the amino acids that will substitute the initial ones was really tedious, because of the fact that for each residue, in each chain, in each antibody of the dataset all possibilities were tested manually. Consequently, residues with exposed side chains, in 'aggregation-prone' regions, were substituted by others with lower tendency to form aggregates, but with similar physicochemical properties and the potential to form aggregates was reduced or even eliminated in some cases.

2.7 Novel Models - Evaluation and Improvements

After the substitutions, novel amino acid sequences were created. The AMYLPRED2 [25] consensus method was used again to predict 'aggregation-prone' regions in the novel amino acid sequences.

The Modeller 9.13 [30] software was used for homology modeling and novel models of the 'mutated' proteins were calculated, utilizing as templates the native structures. The side chains of the 'mutated' residues were examined and optimized, in order to ensure that the side chains are still exposed and to avoid steric hindrance. This was done utilizing Chimera 1.8 [31] and its capability to minimize the energy of the structure. The PROCHECK [32] and WHAT_CHECK [33], from software WHAT IF [34], algorithms were used to check the validity and the quality of the novel models. Special care was taken so that the CDR conformations remain unaltered in the novel models.

3. RESULTS

The sequence alignments of all monoclonal antibodies in the dataset, for the light and the heavy chain separately, are presented in Figs. 1 and 2, respectively. The CDRs for light and heavy chain are named L1, L2, L3 and H1, H2, H3 accordingly. The 'aggregation-prone' regions, that AMYLPRED2 [25] consensus method predicted, are also illustrated and grouped into clusters according to their position in the 3D-structure (Figs. 3A and 3B, BEFORE and Figs. 1 and 2). These clusters were called Cluster1 – Cluster5. Only two specific examples for the monoclonal antibodies RITUXIMAB and ADALIMUMAB are shown in Figs. 3A and 3B.

Performing specific changes of amino acid residues, only in the clusters, 'aggregation-prone' clusters were successfully diminished or even eliminated completely (shown explicitly in Figs. 3A and 3B, AFTER).

The clusters that we grouped the common 'aggregation-prone' regions, according to the 3D-structure of the proteins, are also presented in Table 3 in the column 'Clusters'. The column 'Regions' contains the common 'aggregation-prone' regions, the column 'Chain' the chain of the regions and the column 'Peptides' presents the different peptides that were found in the common 'aggregation-prone' regions. The amino acids that were substituted are illustrated in bold and they are underlined. The column 'After Substitutions' contains the peptides after the substitutions. In some cases, some 'aggregation-prone' peptides were eliminated completely and this is depicted with the symbol (√). In other cases the 'aggregation-prone' peptides were diminished sufficiently and the peptides that remain are presented in the table.

Taking a closer look to the amino acids that were substituted, the amino acids Threonine (THR, T) and the amino acid Serine (SER, S) were mostly substituted. Other amino acids that were considerably substituted by others, were the amino acids Tyrosine (TYR, Y), Valine (VAL, V), Isoleucine (ISO, I) and Leucine (LEU, L). Thus, polar and hydrophobic amino acids which seem to favor aggregation should be substituted by other polar or charged amino acids, considering the experimental aggregation propensities of the 20 natural amino acids [28].

Every 'aggregation-prone' peptide, which belongs to a cluster, with a combination of one or two substitutions, will no longer be 'aggregation-prone'. There are several potential combinations of substitutions that may bring the desirable result. The novel models that are proposed, after the substitutions, will be less 'aggregation-prone' and should not provoke immunological reactions to patients. This remains to be proven by more refined experimental work.

We conclude that, comparing the space-filling models before and after the substitutions, with specific replacements of exposed amino acid residues, only in the clusters that contain common 'aggregation-prone' regions, the potential of antibodies to form aggregates will be reduced or even eliminated. Accordingly, their aggregation propensity should also be reduced without affecting the potency of the bio-therapeutics.

Table 3. Summary of the clusters of the common 'aggregation-prone' regions in the antibodies used for this study

Clusters	Regions	Chain	Peptides	After substitutions
Cluster1	RVTITC	LIGHT	RV**T**I**T**C	√
		LIGHT	RV**T**ITC	√
Cluster1	[FY]T[FL]TISS	LIGHT	FTFTI**S**S	FTFTI**S**
		LIGHT	F**T**LTI**S**S	√
Cluster2	I[VL]LTQ	LIGHT	I**L**L**T**Q	√
		LIGHT	I[VL]L**T**Q	√
Cluster2	[AV]ILS[AV]	LIGHT	[AV]**I**LS[AV]	√
Cluster3	PSVFIF	LIGHT	PSVF**I**F	√
		LIGHT	PSV**F**IF	√
		LIGHT	P**S**VFIF	FI
Cluster3	TASVVCLLNNFY	LIGHT	**T**ASVVCLLNNF**Y**	SVVCLLNN
		LIGHT	**T**ASVVCLLNNFY	SVVCLLNNF
Cluster3	YSLSSVVTV	HEAVY	**Y**SLSSVV**T**V	√
Cluster3	GTQTYICNVN	HEAVY	GTQT**Y**ICNVN	√
		HEAVY	GTQ**T**Y**I**CNVN	√
		HEAVY	GT**Q**TY**I**CNV	√
		HEAVY	**T**Y**T**C	√
Cluster4	A[TV]YYC	LIGHT	A**T**YYC	√
		LIGHT	ATY**Y**C	√
		LIGHT	A**V**YYC	√
Cluster5	[TS]A[VIL]YYC	HEAVY	TA**V**YYC	YYC
		HEAVY	**S**A**V**YYC	√
		HEAVY	A**V**YYC	√
		HEAVY	**T**A**V**YYC	√
		HEAVY	TA**I**YYC	√
		HEAVY	**T**A**L**YYC	A**L**YY
Cluster5	G[TS][TL]VTVS[SA]	HEAVY	GT**L**V**T**VS[SA]	√
		HEAVY	GT**T**VTVS[SA]	√
		HEAVY	**T**LTVS**S**	√
		HEAVY	GS**L**VTVS[SA]	√
		HEAVY	GT**L**VTVS**S**	√
BURIED	GCLVK	HEAVY	GCLVK	GCLVK
BURIED	W[IVL][GSA]	HEAVY	W[IVL][GSA]	W[IVL][GSA]

The clusters of the 'aggregation-prone' regions, the 'aggregation-prone' regions, the chain, the specific peptides for each region (the amino acids that were substituted are in bold letters and underlined) and the final results after the substitutions (the 'aggregation-prone' peptides that were eliminated completely are depicted with (√) and the ones that remain are shown)

4. DISCUSSION

The mechanisms of protein aggregation are very complicated. Protein aggregation and the problems it causes in biopharmaceutics are discussed in detail, in a relatively recent excellent review [16]. The aggregation of a protein in solution is driven by intrinsic and extrinsic factors [29]. The sequences and structures of Fab commercial and non-commercial antibodies were studied in detail by these authors, utilizing the computational tools TANGO [35] and PAGE [36], to identify potential aggregation-prone regions in these antibodies (intrinsic aggregation factors). They discovered in their dataset 2 to 8 aggregation-prone motifs per heavy and light chain pair. Some of these motifs were located in variable domains, mainly in CDRs [29].

In this work, we collected all mAbs against cancer and autoimmune diseases, with solved crystal structures from the PDB [22], and with aid of AMYLPRED2 [25], a consensus algorithm, which combines eleven (11) individual algorithms for the prediction of aggregation-prone sequences in globular proteins (including TANGO), we predicted aggregation-prone regions in the light and heavy chains of these antibodies.

We should mention here that, AMYLPRED2 (and its previous version AMYLPRED) have been used with considerable success, in recent years,

for the prediction of aggregation-prone peptides in amyloidogenic proteins [37-41]. The predicted aggregation-prone peptides have been synthesized and experimentally shown to self-assemble (aggregate) to form amyloid-like fibrils. AMYLPRED2, freely available for academic users, is one of the most successful algorithms for the prediction of aggregation-prone segments (peptides) in globular proteins, as can be seen in the original publication [25].

The idea of predicting aggregation-prone regions of light and heavy chains of mAbs was to try to identify clusters of aggregation-prone regions that could create aggregation "hot-spots" on the surface of these proteins. These aggregation "hot-spots" could drive the mAbs to aggregate. Performing suitably selected mutations on these "hot-spots", we managed to diminish or even completely eliminate them. Special care was taken that the performed mutations did not affect the conformations of the CDRs of the antibodies. Also, care was taken that the mutations were not performed to amino acids contained in aggregation-prone regions, which overlap with CDRs.

5. CONCLUSION

This computational study can be generalized in other monoclonal antibodies that are used therapeutically for other diseases. Work is in progress along these lines (In preparation). It should be emphasized that experiments should be performed to experimentally test the monoclonal antibody variants predicted in this study and verify that have lower aggregation propensities and also ensure that there will be no side effects. A general computational method for a semi-automated optimization of the aggregation propensity of all monoclonal antibodies will be proposed soon (work in preparation).

We hope and anticipate that the combination of predictions, experiments and molecular models of the candidate targets can be applied on novel and existing bio-therapeutics with promising results. Finally, the continuous development and application of rational protein design technology will enable improvements in the efficacy and safety of protein therapeutics.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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